Title: OX40: Structure and function – what questions remain?

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Abstract

OX40 is a type 1 transmembrane glycoprotein, reported nearly 30 years ago as a cell surface antigen expressed on activated T cells. Since its discovery, it has been validated as a bone fide costimulatory molecule for T cells and member of the TNF receptor family. However, many questions still remain relating to its function on different T cell subsets and with recent interest in its utility as a target for antibody–mediated immunotherapy, there is a growing need to gain a better understanding of its biology.

Here, we review the expression pattern of OX40 and its ligand, discuss the structure of the receptor:ligand interaction, the downstream signalling it can elicit, its function on different T cell subsets and how antibodies might engage with it to provide effective immunotherapy.
Highlights:

- The OX40:OX40L interaction is a key regulator of T cell responses
- OX40L is part of divergent class of TNF superfamily ligands
- OX40 signalling can promote Teffector proliferation as well as inhibit Treg function
- Potential immunotherapeutic target for cancer, inflammatory and autoimmune diseases
- Further understanding of context dependent function will improve clinical outcomes
Abbreviations:
ADCC – Antibody-dependent cellular cytotoxicity
APCs – Antigen Presenting Cells
CDC – Complement-dependent cytotoxicity
CRD – Cysteine rich domain
CTL – Cytotoxic T lymphocyte
DP – Double positive thymocytes
FcγR – Fc gamma receptor
iTreg – induced Treg
KO – Knock-out
mAb – monoclonal antibody
MHC – Major histocompatibility complex
NK – Natural killer cells
NKT – Natural killer T cells
nTreg – natural Treg
SP – Single positive thymocytes
TCR – T cell receptor
TLRs – Toll-like receptors
TNFRSF – Tumour necrosis factor receptor superfamily
TNFSF – Tumour necrosis factor superfamily
TRAF – TNF receptor associated factor
Tregs – Regulatory T cells
WT – Wild-type
OX40 expression; When, where, how?

The T cell co-stimulatory receptor, OX40 (CD134), and its interacting ligand OX40L (CD252) are members of the tumour necrosis factor receptors/tumour necrosis factor superfamily (TNFRSF/TNFSF) involved in potentiating T cell responses triggered through the T cell receptor (TCR). Other members of this superfamily include 4–1BB, CD40 and GITR [1, 2]. A key means of co-ordinating signalling through OX40 is by controlling expression of both the receptor and ligand. Both mouse (m) and human (h) OX40 are expressed primarily on activated T cells, including CD4, CD8, T helper cell subtypes; Th1, Th2 and Th17, as well as CD4+ Foxp3+ regulatory T cells (Tregs) (reviewed in [3–5]). OX40 is also expressed to a lesser extent on neutrophils, natural killer cells (NK) and natural killer T cells (NKTs) [6–8]. Unlike other co-stimulatory receptors, such as CD28 and CD27, OX40 is not expressed on naïve T cells; however antigen (Ag) stimulation and subsequent signalling through the TCR results in OX40 expression that peaks between 12h and 5–6 days post Ag stimulation [9–12]. The kinetics of receptor expression are determined by a number of factors including; T cell subset, persistence of antigen as well as immunogenicity, inflammatory environment i.e cytokine milieu, as well as the presence of other co-stimulation [11]. Studies have shown that CD28–B7.1/2 interactions sustain the expression of OX40 on the surface of T cells [10, 11], allowing increased survival and proliferation due to prolonged OX40 signalling. However, CD28 stimulation is not involved in the re-expression of OX40 on effector cells [10, 13], perhaps because these cells are already sensitized to TCR stimulation.

Like OX40, human and mouse OX40L is only induced after stimulation – in this case through signals such as CD40–CD40L interactions, Toll-like receptors (TLRs) and inflammatory cytokines such as thymic stromal lymphoprotein (TSLP) [14, 15]. OX40L has been detected on the surface of antigen presenting cells (APCs) 1–3 days after antigen encounter [16]. It was thought previously that OX40L
expression was limited to professional APCs such as activated B–cells, dendritic cells and macrophages, however studies have revealed that it is also expressed on NK and mast cells [17–21] as well as structural cells such as smooth muscle cells and vascular endothelial cells in the presence of inflammatory cytokines [22, 23]. These findings illustrate how the OX40–OX40L interaction is not confined to specific tissues or organs but that its effects have the potential to be widespread throughout the body.

**Structural analysis of the OX40:OX40L interaction:**

Insight into the structural basis of the OX40–OX40L interaction was gained by solving the crystal structure of hOX40 in complex with both murine and human ligand [24], deposited within the protein data bank (PDB) as 2HEY and 2HEV, respectively. OX40 is a type 1 transmembrane glycoprotein composed of ~275 amino acids dependent on species (human–accession number: NP_003318.1, murine–accession number: CAA59476.1 and rat–accession number: P15725.1). OX40 has an apparent molecular weight of 50kDa, owing to N–linked glycosylation of the 30kDa polypeptide chain [25, 26] at positions N146 and N160. Solving the crystal structure of hOX40 (277 amino acids) in complex with both murine and human ligand nearly 20 years after its initial discovery allowed for a more in–depth understanding of this interaction and highlighted some key differences in this receptor–ligand interface which are not seen in other TNF family members such as TNFR/LT [27] and DR5–Apo2L/TRAIL[28, 29].

The crystal structure of hOX40 shows that the receptor conforms to a relatively conventional multi–domain TNFR structure consisting of an extracellular ligand binding domain formed of 4 cysteine rich domain (CRD) repeats. CRD’s 1 and 2 clearly possess A1–B2 modules. However, our interpretation of the module formation of the 3rd and 4th CRD is different than that proposed in the original structural paper. Sequence alignment with a typical A1–B2 module indicates that the 3rd CRD is truncated and possesses just an A1
module, with the 4th CRD possessing an A1–B1 module, rather than a full 3rd module and truncated 4th module as originally proposed. [24, 30, 31] (Figure 1A). OX40 CRD1 and 2 are relatively conserved with respect to other TNFRSF members and form a rigid unit equivalent to the structures seen in other TNFRs, such as TNFR1 (structural similarities reviewed in [32]). In contrast, there is more rotational freedom seen between CRD3 and CRD4 that is not seen in TNFR1, highlighting the structural diversity in this family of receptors (Figure 1B).

Both murine and human OX40L reveal substantial differences compared to other TNFSF ligands [24]. OX40L only possesses 15% sequence identity in relation to other TNF family members [33] and only 40% sequence identity between the mouse and human ligands; far less than other TNFSF orthologs. Nonetheless, the same structural features are maintained. However, upon trimerisation with its receptor, key structural differences are seen in comparison to other TNFSF members which are highlighted in the crystal structure; these include the angle between the trimer axis and each monomer being ~15° wider than conventional TNFSF members and the trimer interface being smaller (Figure 1C). These distinctive sequence and structural differences make it clear why both murine and human OX40L are described in the divergent category of TNFSF as opposed to the conventional or EF–disulfide–containing categories [24, 30].

Two amino acids, F180 and N166, are conserved between murine and human OX40L and make similar contributions to receptor binding [24]. Mutational analysis identified the importance of these residues and revealed that unlike other TNFSF members, OX40L makes contact with the receptor over at least 2 areas and not just one ‘hot spot’ [24] (Figure 1D). This finding agrees with the crystal structure data detailed above; a wider angle between each monomer and the trimer axis suggests a greater potential for the ligand to make multiple contacts with the receptor.
The solved hOX40–hOX40L complex [24] is 80Å long, which along with the linker region connecting the extracellular domain to the transmembrane domain, which is 40 amino acids in length, indicates that the complex can connect cells which are 100–150Å apart; a common distance seen between other receptor ligand interactions such as the CD2–CD48 adhesion complex (134 Å) [34]. A similar spanning distance is predicted for the TCR–major histocompatibility complex (MHC)–peptide complex and in line with the kinetic segregation model [35] indicates that these co–receptor molecules are the appropriate size to enter the ‘close contact zones’, created between cells via ligand:receptor interactions, excluding bulky glycocalyx elements resulting in increased half–life of phosphorylated species [36]. This finding suggests that hOX40–hOX40L interactions facilitate direct cell–cell communication and hence provides evidence that this contact is responsible for the functional effects seen in in vitro and in vivo.

In recent years, alongside many other T cell regulatory receptors, OX40 has become a target for immunotherapy using monoclonal antibodies (mAb) [37, 38]. One potential mode of action for these reagents is by mimicking ligand and eliciting agonistic receptor signalling to promote T cell activation, for example in the presence of immunosuppressive cancers. There are several ways to activate the OX40 pathway (Figure 2A) and many questions remain relating to how OX40L and anti–OX40 mAbs may modulate this. For instance, whether OX40 can signal effectively solely through bivalent antibody stimulation and if so does this result in a full signal transduction downstream of the receptor (Figure 2Ai)? Studies with hOX40L suggest that higher order oligomerisation is required for effective signalling to be induced and thus merely bivalent mAb stimulation of OX40 is unlikely to be optimal or even effective [39]. Given the multi–domain structure of OX40, and known contact points of the ligand (Figure 1D), certain anti–OX40 mAbs are likely to be capable of binding to the receptor in the presence of the ligand.
However, it is not yet clear how concurrent ligand and mAb binding would effect signalling, if OX40L binding is a prerequisite for signalling or indeed if mAb binding alone in some circumstances/at certain epitopes is sufficient for full signalling (Figure 2Aii and iii)? Also, how do Fc gamma receptors (FcγR) modulate antibody induced OX40 signalling (Figure 2Aiv)? Lastly, in addition to agonists, it is clear that antagonistic mAbs, which can block OX40 signalling may also have virtue in immunotherapy – for example to block hyperactive T cell pathologies [40]. What affords agonistic or antagonistic mAbs their opposing activities is not yet clear. Further insight from structural analysis of antibody receptor interactions and binding epitopes as well as more detailed examination of downstream signalling will be important for understanding the requirements necessary to elicit the optimal downstream effects of OX40–OX40L interactions.

**Signalling through OX40:**

A number of signalling pathways have been identified downstream of OX40–OX40L interactions including those mediated by PI3K/PKB, NF-κB and NFAT that account for the reported functional consequences of T cell division, survival and cytokine production (Figure 2B) [41, 42]. Nonetheless, delineation of signalling directly downstream of OX40 is limited as the majority of studies have been done in conjunction with TCR signalling (because naive T cells do not express OX40 until they have been activated through the TCR) and have thus principally showed how OX40 can augment signalling downstream of TCR – MHC/peptide interactions.

The cytoplasmic tail of OX40 contains a QEE motif characteristic of many TNFR family members that allows binding to TNFR–associated factors (TRAFs) [42, 43]. Formation of trimeric receptor:ligand complexes clusters the cytoplasmic domain of OX40, creating docking sites for TRAF adaptor proteins, linking receptor activation to various signalling pathways. Co-immunoprecipitation studies in mammalian cells have shown that the QEE motif in the cytoplasmic domain of
h/mOX40 is able to recruit TRAF 2, 3 and 5 [43]. Furthermore, deletion mutants showed that the GGSFRTP1 sequence immediately upstream of the QEE motif in humans is required for association of TRAFs 1,2,3 and 5 [44]. It was additionally shown that TRAF 2 and 5 were able to activate the NF–κB signalling pathway whereas TRAF 3 was likely to have an inhibitory effect [44]. TRAF 2, 3 and 5 were shown to be able to directly interact with the inhibitor of NF–κB α–subunit (κ–Bα), IκB kinase complex catalytic subunit IκKβ and NF–κB inducing kinase (NIK) demonstrating that OX40 can regulate both the canonical (NF–κB1) and non–canonical (NF–κB2) signalling pathways [4, 44, 45]. Likely through its regulation of the NF–κB pathways, OX40 activation has been shown to increase the expression of a number of anti–apoptotic proteins including Bcl–2, Bcl–xL and Bfl–1 (A1) causing suppression of apoptosis and enhanced cell survival [11].

As mentioned above, OX40 activation and its consequent downstream signalling has also been shown to augment the signalling downstream of the TCR, mainly through effects on the PI3–K/PKB pathway. Song et al. demonstrated that activation of PKB was not sustained in OX40 knock–out (KO) T cells with these cells subsequently undergoing extensive cell death post activation suggesting that OX40 is central to T cell longevity through combined effects on PKB from the TCR and OX40 after antigen encounter [46]. Furthermore, synergy between TCR and OX40 signalling increases the expression of survivin and Aurora B kinase through sustained PKB activation [47, 48]. These two proteins function together to promote the activity of cyclin dependent kinases allowing for G1 to S phase progression in the cell cycle and maintenance of mitosis in T cells [47, 48]. Additionally, OX40 ligation in conjunction with TCR signalling can increase calcium influx and enhance NFAT activation. This pathway is involved in the production of cytokines such as IL–2, IL–4, IL–5 and IFN–γ [49]. Thus, signalling downstream of OX40 has the potential to augment proliferation, suppress apoptosis and induce greater cytokine responses from T cells; all of which are functional outcomes that
agonist OX40 antibodies have the capacity to elicit when being used as forms of immunotherapy.

**Function of OX40:**

The most recognised function of OX40 is to enhance proliferation and survival of CD4 and CD8 T cells through the pathways described above. These activities were first shown *in vitro* whereby addition of OX40L or agonistic anti–OX40 mAb resulted in proliferation of CD4 T cells [33, 50, 51]. The development of more sophisticated *in vitro* systems alongside the generation of OX40 and OX40L KO animals, plus the use of agonist and blocking antibodies *in vivo* revealed the full spectrum of effects induced by engaging OX40. For example, it is now well established that OX40 interaction with ligand enhances sustained proliferation and optimal clonal expansion of CD4 T cells in primary and secondary response to antigen; as well as promoting effective memory generation [9, 10, 15, 52, 53]. OX40 has also been shown to have effects on cytokine production, which depending on context can lead to differentiation of CD4 T cells into either Th1 or Th2 subsets whilst also demonstrating a role in IL–17 production and regulating Th17–mediated diseases [4, 14, 54, 55]. The engagement of OX40L with OX40 on naïve CD4 T cells (after its TCR–mediated upregulation) preferentially leads to the differentiation of Th2 cells as a result of autocrine IL–4 production [14, 49, 56]. However, the presence of IL–12 or Type I interferons can override this divergence and lead to Th1 differentiation [14, 57, 58]. It has also been demonstrated in a mouse model of asthma that the absence of OX40 results in an impaired Th1 response but the Th2 response remains normal [59, 60] suggesting Th1 development is more reliant on OX40 and its signals. This demonstrates the ability of OX40–OX40L interactions to influence the local cytokine environment and hence the nature of the T–helper cells produced. The role of OX40 on Th17 cells is less clear. It has been shown to be involved in IL–17 and IL–23 cytokine production in both *in vitro* and *in vivo* models leading to the
differentiation and expansion of Th17 helper cells [54, 61]. However, data also suggest that OX40 engagement antagonises Th17 development by inhibiting IL-17 production [55, 62]. Th17 cells have been implicated in a number of autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis because of their involvement in the inflammatory response [63–66]. As OX40–OX40L interactions are being investigated as a potential point of therapeutic intervention in autoimmunity, it is important that further work clarify its role on Th17 cells, particularly with regards its potential to be disease- and inflammation-context dependent.

The understanding of the role of OX40 on CD8 T cells has lagged behind that of CD4 T cells mainly because initial studies using OX40 KO mice challenged with virus suggested that OX40 had minimal impact on the cytotoxic T lymphocyte (CTL) response [67, 68]. However, subsequent studies using TCR transgenic mice revealed that CD8 T cells are also affected by OX40–OX40L interactions [12, 69]. The results observed were similar to that seen during the CD4 response i.e. in the absence of OX40, TCR transgenic CD8 T cells showed defective expansion, decreased survival and poor memory generation [12, 41, 69, 70].

Overall, the data indicate that OX40 signalling promotes robust immune responses and therefore agonistic antibodies are a potential therapeutic target for cancer whilst antagonistic antibodies may prove useful in the treatment of autoimmune and inflammatory diseases. One potential caveat to this simplistic approach is the complexity relating to how OX40 is regulated on different T cell subsets; in particular the role of OX40 in Treg development and function.

**OX40 on Tregs**

OX40 is expressed constitutively on murine Tregs unlike naive T cells. It has also been reported that OX40 is more highly expressed on Tregs, particularly on those isolated from tumour sites [71–73].
Therefore, it is possible that antibody therapy may target these cells preferentially, and so understanding how OX40 modulates Treg biology is vital in directing how we use these reagents therapeutically.

**Role of OX40 in Treg Development**

Initial studies in OX40 KO mice revealed a defect in Treg development which was more apparent in young mice [74, 75]. The frequency of thymic Tregs in the double positive (DP) population was unaffected with the defect becoming more apparent at the single positive (SP) stage, suggesting that lineage commitment is not compromised in these mice but rather maintenance, survival or proliferation is affected [74]. As thymocytes transition from the DP to the SP stages, they undergo significant selection pressures with the majority of DPs undergoing cell death either through “neglect” or negative selection (reviewed in [76]). Both proliferative and mild survival defects have been reported in OX40 KO mice [74, 75], therefore it may be that at different stages of development these processes contribute to the Treg defects that are observed.

The fact that the loss of Tregs in OX40 KO mice was more apparent in younger mice and occurred in the absence of autoimmunity suggests that any defect was subtle [75, 77]. This conclusion was further supported by bone marrow chimera studies which showed defects under competition settings with OX40-competent wild-type cells whilst overexpression of mouse OX40L via an OX40L transgene resulted in increased numbers of Tregs [74, 75, 78, 79]. It should be noted that there were experimental caveats to the interpretation of the data in the OX40L overexpressing mice; with strong OX40L expression observed on the Tregs in these mice and undetectable levels on wild-type mice [75]. OX40 expression was also reduced on the Tregs in these mice suggestive of a compensatory effect in response to OX40L overexpression, which may have impacted on subsequent development [75]. Later studies using OX40 KO Foxp3 GFP knock-in mice appeared to show little–no defect on Treg
development [80–82], although it was suggested that there may be a
defect in Treg homeostasis in this model which is context dependent
as there were reduced numbers of Tregs in non-lymphoid organs such
as the colonic lamina propria [81]. More recently the subtle role for
OX40 in Treg development was supported by data showing that
members of the TNFR family act cooperatively to produce the full Treg
repertoire [78]. Mahumd et al. used conditional KOs of GITR, TNFR2
and OX40 to show that individually these genes had limited impact on
the generation of Tregs but that the combination of all three TNFR KOs
resulted in significant inhibition of natural Treg (nTreg) development
[78]. In addition, it was shown that OX40 signalling altered
responsiveness of Tregs to IL–2 signalling, possibly via a
miR155/SOCS1–dependent mechanism [78]. This agrees with data
showing that OX40 KO T cells are defective in their response to IL–2
signalling despite normal levels of its receptor, IL–2R (CD25) [74, 79].
As IL–2 is required for Treg homeostasis, [83, 84] this is a possible
mechanism whereby OX40 contributes to the development of nTregs.
Thus taken together the majority of data supports a fairly minor, and
as yet ill–defined, role for OX40 in Treg development.

In contrast to the minor requirement for OX40 in nTreg
development, studies show that OX40 signalling may play a more
central role in the development of induced Tregs (iTregs). In vitro
models of Ag–induced or TGFβ–mediated conversion of CD4 effector T
cells both show enhanced conversion of OX40 KO cells and an
inhibition of conversion when agonistic OX40 antibody is included in
cultures containing WT effectors [80, 85, 86]. Furthermore, studies
with OX40L also show that OX40 signalling is detrimental to the
development of a range of iTregs [86–88]. However, in the OX40L Tg
mice, more effector memory cells were observed which were resistant
to conversion to Tregs by TGFβ [86]. Likewise, a study using an OX40
agonistic antibody revealed an increase in the polarising cytokines IFNγ
and IL–4 and that when these were neutralised, OX40 signalling
actually enhanced the development of iTregs [89]. These studies raise
the question as to whether OX40 signalling directly alters the ability of iTregs to develop or whether it modifies the phenotype of the effector population and/or cytokine milieu. In addition to altering the cytokine environment and subsequent effects on the conversion of effectors into iTregs, another possible mechanism by which OX40 signalling inhibits iTreg development is through modulation of Foxp3 expression. Several groups have reported that OX40 signalling is capable of downregulating Foxp3 expression [79, 80, 85]. However studies in OX40 KO mice have shown no changes in Foxp3 expression compared to wild-type mice suggesting that OX40 effects on Foxp3 expression are context dependent [74, 80]. One possible way OX40 could affect Foxp3 expression is through inducing alterations in the binding of pSmad3 and Stat3 to the Foxp3 promoter [87, 90]. Another possibility is indirectly through modulation of the cytokine environment. For example, OX40 drives IL-4 and IFNγ production which could potentially synergize with APC-produced IL-6 to block Foxp3 maintenance [41, 91]. OX40-mediated inhibition of iTreg development has also been observed in an in vivo model of airway tolerance [91]. Likewise, when a tolerising protocol was used to induce Tregs in vivo, conversion was inhibited in OX40L Tg mice [80].

Similar to mouse studies, OX40 signalling has been shown to be detrimental to the induction of human iTregs [92, 93]. Agonistic OX40 antibodies were shown to block both the generation of IL-10 producing regulatory cells as well as those induced by TGFβ [92].

In summary, it appears that in mice at least, OX40 has a minor role in the development of nTregs but that in both mice and humans a more substantial, negative, influence on iTreg development is seen. Differences in how OX40 regulates these Treg populations are perhaps not surprising given the varied mechanisms/signalling pathways involved in their generation and hence allow for the possibility that OX40 could have divergent roles in each cell type.
**Role of OX40 in regulating Treg function:**

How OX40 regulates Treg function is also controversial and complicated by the need to distinguish the effects on Tregs from effector CD4s. OX40 KO Tregs display a mild defect in suppressive capability *in vitro* and reduced capacity to suppress mast cell degranulation [42, 75, 94]. Likewise *in vivo*, Tregs are shown to require OX40 in order to fully suppress the induction of colitis in adoptive transfer models [74, 81]. As with the initial controversy over a role in Treg development, OX40 KO Tregs have subsequently been shown to be fully functional [80]. Even where studies have attempted to delineate effects on Tregs versus effector cells in *in vitro* suppression assays there are conflicting results with the only apparent difference being whether cells have been isolated from OX40 KO or OX40 KO Foxp3 GFP knock-in mice [75, 80]. Furthermore, it is possible that the requirement for OX40 on Tregs in the colitis model is in part due to the lymphopenic environment the cells are transferred into as homeostatic proliferation was found to be defective in OX40 KO Tregs [74, 75]. Nevertheless, studies where signalling is provided by agonistic anti–OX40 mAb or OX40L overexpressing APCs have consistently shown inhibition of Treg function both *in vitro* and *in vivo* [73, 79, 80, 95].

There are several ways that OX40 signalling could alter the functionality of Tregs. One possibility is that inducing their proliferation can lead to loss of Treg suppressive activity under certain conditions. However, it should be noted that Tregs *in vivo* are known to proliferate extensively without losing their suppressive capacity (reviewed in [96]). The use of agonist anti–OX40 antibody (OX86) or APCs overexpressing OX40L resulted in clear proliferation and expansion of the mouse Treg population both *in vitro* and *in vivo*, however other studies using the OX86 antibody failed to induce Treg proliferation [75, 79, 80, 97]. Furthermore, the combination of OX86 and IL–2 was shown to promote Treg expansion whilst maintaining function, resulting in improved survival following transplantation of
fully mismatched heart allografts [79], further emphasising the context dependent relationship between Treg expansion and function.

OX40 signalling has been shown to be important for the survival of effector T cells (reviewed in [4]) and this could also be true for Tregs. In a depletion model used to induce tolerance, fewer CD4 effectors and Tregs survived in an OX40 KO background suggesting it influences survival in both subsets [98]. However, this interpretation may again be complicated by the fact that the model induces lymphopenia with the caveats discussed above. Further evidence both for and against the importance of OX40 in regulating apoptosis in Tregs, has come from studies using OX40 KO Tregs [81, 97] and OX40 stimulation [80], respectively, indicating that its role in regulating survival is context dependent.

In addition to regulation through OX40 signalling, in the context of mAb mediated immunotherapy, another possible mechanism for suppression of Treg functionality is through their mAb-mediated depletion. Evidence for this mechanism are mixed. Although some tumour studies report clear depletion of Tregs, the majority do not [71–73, 75, 97]. Whether these discrepancies are simply a result of differing levels of Tregs, varying amounts of OX40 on the Tregs, or altered immune suppression in the different tumour models is currently unclear.

**Role of OX40 on Human Treg function:**

As with murine Tregs there are also conflicting data surrounding the role of OX40 on human Tregs. A study looking at Tregs isolated from chronic hepatitis C patients showed that OX40+ Tregs were more suppressive than their OX40− counterparts and that additional OX40 signallling, provided by OX40L+ APCs, did not alter suppressive function [93]. However, another group showed that anti–OX40 antibodies which induced strong CD4 effector and memory proliferation, also potently blocked Treg mediated suppression [92]. In this particular setting it was not possible to discern whether the
effects were on effector CD4s or Tregs and therefore whether one of these was dominant. However, pre-pulsing Tregs with agonistic OX40 antibody did reduce suppressive capacity suggesting, as with the majority of murine studies, that additional OX40 signalling is detrimental to Treg function [92]. It should be noted that pre-pulsing effectors in the same way also made them less sensitive to Treg mediated suppression, again similar to results reported for murine cells.

The anti-OX40 mAb discussed above induced proliferation of both naive effectors and Tregs in a dose-dependent manner [92]. Likewise, the use of artificial APCs expressing OX40L or M2-like monocytes and macrophages which had upregulated OX40L were shown to be capable of expanding Tregs without a loss of suppressive function [93, 99]. Thus, in these settings OX40 signalling may ultimately improve Treg function through the expansion of Treg numbers. However this may be offset depending on context, as akin to murine Tregs, high doses of antibody reduced Treg viability via an apparently apoptotic process [92].

**OX40 as an immunotherapeutic target**

The information detailed above highlight the potential ways in which OX40 might serve as an effective target for immunotherapy. Modulating OX40 signalling and/or deleting different T cell subsets has the potential to mediate both immune suppression for autoimmunity and immune stimulation for anti-cancer therapeutics. The OX40:OX40L interaction has been targeted in a wide range of models of inflammation, cancer and autoimmunity where the predominant effector population is T cells. However, it is important to note, as discussed above, other cell populations also express OX40 and may have subtle but important roles in regulating any therapeutic modalities.

**OX40 in autoimmune and inflammatory diseases:**
In an inflammatory or autoimmune environment the cells of the immune system are typically hyperactive and/or misdirected towards the tissues of the host. It has been shown in mouse models of EAE, asthma, colitis, arthritis and GVHD that OX40 plays a role in the exacerbation of disease and thus by blocking the OX40:OX40L interaction, disease symptoms can be ameliorated [19, 38, 100–106], through changes in CD4 effector functions. As discussed earlier, OX40 expression is highest on Tregs and CD4s and hence the fact that the therapies exerted their effects on CD4s suggests that expression may well be a determining factor as to which cells are targeted.

**OX40 in cancer:**

OX40 as a therapeutic agent has been investigated in a number of preclinical tumour models; using both anti–OX40 mAbs and OX40L–Fc fusion proteins. OX40 therapy has been shown to cause tumour regression and delayed tumour growth mainly in immunogenic models [3, 5, 37, 38, 73, 106–109]. OX40 dependent anti–tumour immunity required the expansion of CD8 and CD4 T cells, with a proportion of mice showing evidence of strong memory responses sufficient to provide resistance upon tumour re–challenge [108, 110]. As discussed above, recent studies have showed that in certain models Treg depletion is also an important component of OX40 therapy. In the first-in-human clinical trial with an anti–human OX40 agonist antibody promising results were seen, where 12 out of 30 patients showed evidence of tumour regression after just one cycle of treatment in a number of solid tumour types [111]. Nevertheless, despite its promising results anti–OX40 monotherapy was insufficient to treat all cancer types or all patients. However, further mouse model studies using anti–OX40 in combination with other monoclonal antibodies, chemotherapy and cytokines have all shown increased therapeutic effects in comparison to monotherapies and thus provides an encouraging direction for clinical development (Reviewed in [37]).
Clinical considerations:

In order to best augment the capabilities of anti–OX40 antibodies, their key mechanisms of action should be clearly identified. On the basis of our current understanding, OX40 mAb have the potential to deliver anti-cancer immunotherapy through the following means: (Figure 3) direct stimulation of effector T cells (CD4 and CD8+); signalling inhibition of Tregs and deletion of Tregs; or a combination of all 3. Many of the preclinical models that have shown a therapeutic effect of anti–OX40 (reviewed in [37, 38, 106]) have used the same antibody (OX86) and have implicated different mechanisms including the potential for a dual capacity of improving effector function at the same time as inhibiting Treg function. The principal mechanism may in fact vary between different diseases, different cancers and even different individuals and will vary depending on a number of factors including expression profiles on the different T cell subsets and the relative number/ratio of Treg: effectors.

Therefore, although initially, much of the data relating to OX40 may have been viewed as contradictory, it now seems likely that the individual circumstances present within each model alters the nature of responses observed. For example, given the high level of heterogeneity of Treg infiltration and activity even within defined tumour sub-types it seems likely that these requirements may even vary on a patient by patient basis. This situation presents clear challenges, not least with the selection of mAb format and isotype.

Selection of isotype:

The selection of isotype for a therapeutic mAb is critical as it defines how the mAb will engage with the effector mechanisms of the immune system [112–114](reviewed in [115–119]). Defined by the Fc (Fragment crystallisable) portion of an antibody, the isotype is responsible for dictating the strength of engagement of the IgG with C1q and also the Fcγ receptors (FcγR) amongst other molecules [117].
Certain isotypes such as the murine IgG2a and human IgG1 are optimal for engaging activatory FcγR and eliciting target mediated deletion [113, 115, 120–122], whereas murine IgG1 and human IgG2 are sub-optimal and are typically more effective at precipitating the receptor clustering required to drive TNFR signalling. In the case of murine IgG1 this is largely by engaging the inhibitory FcγRIIib, which provides enhanced crosslinking of the antibody:receptor complexes [114, 122–125]. For human IgG2 by contrast, this appears to be at least partially independent of FcγR and relates to the particular properties of the IgG2 hinge [115, 126]. As a result, deletion of Tregs would be favoured by hIgG1, whereas hIgG2 may favour agonistic responses. It remains to be seen whether a single antibody format can deliver both of these activities. Bulliard et al showed that the OX40 antibody OX86 (a rat IgG1) required the activatory FcγR receptors for therapeutic benefit [71]. As expected, the mlgG2a isotype showed greater depleting capacity, resulting in an increased CD8:Treg ratio compared with the rat IgG1 agreeing with recent suggestions that the correct selection of isotype will improve therapeutic effects. Several Fc engineering approaches have recently been shown to augment the anti-tumour activities of TNFR antibodies by altering their interaction with FcγR, and specifically FcγRIIib [127]. Zhang et al demonstrated that a number of different point mutations (previously described [127, 128]) enhanced binding of humanised anti–OX40 antibodies to FcγRIIib, increasing FcγRIIib crosslinking mediated agonistic activity, as well as altering effector functions in an isotype dependent manner [129].

However, selection of antibody isotype only has any relevance if the required FcγRs are present. With respect to oncology different tumours have different microenvironments and infiltrates which will influence the FcγR availability. Hence a strategy based on Treg depletion via a hIgG1 may have limited effect in some tumours due to a lack of appropriate FcγRs. Likewise, the tumour location and even the route of administration will likely alter the FcγRs an antibody may encounter and hence the overall outcome. It was notable that in some
of the tumour models reporting Treg depletion with an OX40 antibody, the antibody was administered intratumourally resulting in local immunomodulation that proved to be more therapeutically beneficial than systemic administration [72].

To date, the most impressive therapeutic benefits seen with OX40 have involved combination therapy. Hence, another important consideration will be what is combined and in these combinations which isotypes are employed.

Thus, the future for OX40 therapy may be bright, but there are still several questions which remain to be answered. What is the key effector mechanism in vivo in humans? Does this vary with disease and individual? Is it possible to develop an antibody capable of deleting one cell subset whilst expanding another? It may well be that these answers lie in further dissecting tumour microenvironments and FcγR interactions. Once these are better understood the possibility of mAb engineering is available to provide tailored medicine for particular disease settings and individual patients.

Declaration of interest
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**Figure Legends:**

**Figure 1. Structural comparison of OX40:OX40L with other TNFRSF members.** A) hOX40 (PDB:2HEV) displayed as a molecular surface with CRD1, CRD2, CRD3 and CRD4 coloured yellow, orange, magenta and red, respectively. The sequence alignment of the four individual CRD’s of hOX40 are displayed in comparison to a typical A1–B2 module and illustrates the specific module formation in each domain. A1 modules are shown in blue, B2 modules in orange and B1 modules in green. Disulfide connectivity is indicated by lines above the sequence and labelled S–S bridges. B) Superposition of hOX40 (red) and TNFR1 (PDB:1TNR) (blue) showing the similarities in structural arrangement of domains CRD1 and CRD2, as well as the flexibility in the CRD3 and CRD4 domains (CRD3 and CRD4 are boxed). C) Comparison of trimer formation between hOX40–OX40L and hCD40–CD40L (PDB:3QD6). Ligand is shown in green and receptor in blue. The black dot represents the trimer axis and the dashed lines the angle between each monomer from this axis. The trimer is orientated such that the trimer is viewed from the point of the ligand–expressing cell membrane. D) hOX40–OX40L interaction. Molecules are shown as a mesh surface with CRD domains coloured as in Figure 1a. OX40L is in green. The arrow represents the spanning distance of the OX40–OX40L complex (80Å). The monomer is orientated such that the ligand–expressing and receptor–expressing cell membranes are at the top and bottom, respectively.

**Figure 2. Mechanisms of OX40 activation and the downstream signalling effects.** A) Potential mechanisms of engagement of OX40 by OX40L and monoclonal antibodies (mAbs). i) Bivalent mAb binding with monomeric OX40. ii) Bivalent mAb binding with pre–clustered OX40. iii) OX40L–assisted mAb–mediated signalling. iv) Fc gamma receptor (FcγR)–assisted mAb signalling (which would be dependent upon the isotype and specific FcγR). B) Known signalling pathways
downstream of OX40. OX40–OX40L interactions are able to directly activate the NF–kB signalling pathway as well as augmenting signalling pathways downstream of the TCR, for example PI3K/PKB and NFAT. These signalling pathways result in effects linked to sustained survival and increased proliferation as well as cytokine release.

**Figure 3. Potential modes of action of anti-hOX40 mAbs as cancer immunotherapeutics.** Current understanding provides evidence for 3 mechanisms of action; 1) direct stimulation of effector T cells (CD4 and CD8+) using an agonist mAb causing enhanced proliferation and survival; 2) signalling inhibition of Tregs provided by an agonist mAb resulting in reduced suppressive function, and lastly; 3) deletion of Tregs with a deleting mAb causing a reduced suppression of effector cells. Combination of all 3 mechanisms acting synergistically may also be possible.
Figure 1

A1B2

--CREKQY-----LINSQC--CSLCQPQGQKLVS--CTEETETEC--

CRD1

LHCVGDTY------PSNDRC--CHECRPGNGMVSR--CSRSQNTVCR

CRD2

--PCGPGFY--NDVVSKPKCKPCTWCNLRSGSERKQLCTATQDVTVC--

CRD3

--RCRAGTQLDSYKPGVDC--

CRD4

--PCPQGHF-----SPGDNQACKPWTN--CTLAGKHTLQFASNSSDAICE--

Blue = A1
Orange = B2
Green = B1

S-S bridges

Ligand-expressing membrane surface

Receptor-expressing membrane surface
Figure 2

A

B

- MHC
- OX40L
- TCR
- OX40

- PI3-K
- PKB/AKT
- Cell survival and cell cycle progression
- TRAF 2 + 5
- Suppression of apoptosis
- Ca2+
- NFAT
- Cytokine production and cell proliferation
Agonist antibody:
- Enhanced survival
- Increased proliferation
- Enhanced/prolonged effector function

Deleting antibody:
- Loss of Treg cells
- Reduced suppression of effector cells

Tregs:
- Inhibition of suppressive activity
- Reduced suppression of effector cells

Effectors: